PA 1166898

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# *PROVISIONAL APPLICATION FOR PATENT COVER SHEET*

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

| Docket Number | 26382 | Type a plus sign (+) inside this box -> | + |
|---|---|---|---|

**INVENTOR(s) / APPLICANT(s)**

| LAST NAME | FIRST NAME | MIDDLE INITIAL | RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY) |
|---|---|---|---|
| GAZIT | Ehud | | Ramat Hasharon, Israel |

**TITLE OF THE INVENTION (280 characters max)**

**A NOVEL METHOD FOR THE INHIBITION OF AMYLOID FIBRILS FORMATION BASED ON ALPHA-AMINOISOBUTYRIC ACID SUBSTITUTED ANALOGUES**

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|---|---|---|---|---|---|

**ENCLOSED APPLICATION PARTS** *(check all that apply)*

| | | | | |
|---|---|---|---|---|
| ☑ Specification | *Number of Pages* | 13 | ☑ Applicant is entitled to Small Entity Status | |
| ☑ Drawing(s) | *Number of Sheets* | 5 | ☑ Other (specify) *ASSIGNMENT* | |

**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT** *(check one)*

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| ☐ A check or money order is enclosed to cover the filing fees | | FILING FEE AMOUNT ($) | $ 80.- |
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

☐ Yes, the name of the US Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date June 26, 2003

REGISTRATION NO. (*if appropriate*) 25,457

TYPED or PRINTED NAME SOL SHEINBEIN

☐ Additional inventors are being named on separately numbered sheets attached hereto

## *USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT*

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
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07/01/2003 WABDELR1 00000142 501407 60483180
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PTO-1556
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# A NOVEL METHOD FOR THE INHIBITION OF AMYLOID FIBRILS FORMATION BASED ON α-AMINOISOBUTYRIC ACID SUBSTITUTED ANALOGUES

**Inventor: Ehud Gazit**

**Abstract**

Efficient inhibition of amyloid fibrils formation is of a key medical importance. Here, we present a new approach to inhibit amyloid fibrils formation. The method is based on a minor modification of a molecular recognition motif with the α-aminoisobutyric acid (Aib). The rational behind the use of this specific modification is the immense structural restriction that is imposed by the Aib amino-acid. When a highly amyloidogenic peptides that contains the molecular-recognition motif of the islet amyloid polypeptide (IAPP) were modified with the Aib amino-acid, no β-sheet structural induction and no fibrils formation could be observed. Furthermore, when Aib-modified peptides were added to solutions that contain full-length IAPP, a significant inhibition in amyloid fibrils formation was clearly observed. Taken together, our approach provides a new tool to inhibit amyloid formation.

**Introduction**

Amyloid fibrils formation is initiated by a site recognition event (Dobson, 2001; Gazit, 2002a-c; Rochet and Landsbury, 2000). We have previously identified a new region within the IAPP molecule that seems to play a major role in this process (Mazor et al., 2002). The region IAPP(14-20) has a higher affinity to the full-length IAPP than does the widely studied region IAPP(23-29). We chose this domain as a target for inhibitors to bind to, and designed peptides based inhibitors, which are directed to this domain.

We used the design approach by which the native peptide domain serves as a template for small chemical modifications, which maintain the recognition properties of the fragment but abolish the ability to aggregate into amyloid fibrils. By that, when added to a solution of IAPP, the new peptides will bind to the recognition domain, block it and prevent its aggregation.

One way to cancel the aggregation ability of the peptides is to insert elements that are β-sheet breakers, that way the peptides wont be able to display a β-sheet conformation by which the monomers are stacked together to form fibrils (Soto et al., 1998; Findeis et al., 1999). For that purpose we introduce a new methodology of using the α-aminoisobutiric acid residue (Balaram, 1999). The α-aminoisobutiric acid (Aib) is an unnatural amino acid that contains two methyl residues attached to $C_\alpha$ of the carboxylic group (Balaram, 1999). Unlike natural amino acids, this molecule does not have a hydrogen atom attached to the $C_\alpha$, this affects widely its sterical properties regarding the $\phi$ and $\psi$ angels of the amide bond. Wile alanine has a wide range of allowed $\phi$ and $\psi$ conformations, Aib, which is a α-methylated alanine has limited $\phi$ and $\psi$ conformations. Its conformational map is derived by the superposition of the Ramachandran plots of L-alanine and D-alanine (Scheme 1A). As one can see, the allowed angels are limited to small regions and are clearly much more suitable for a α-helix conformation than a β-strand conformation. Hence, this molecule can play the role of preventing β-sheet conformation. Moreover, a comparison of the Ramachandran plots for Aib and proline shows that Aib is a better potent β-sheet breaker than is proline (Scheme 1A).

**Experimental procedures**

*Peptides Synthesis* - Peptide synthesis using solid-phase methods was performed by Peptron, Inc. (Taejeon, Korea). The correct identity of the peptides was confirmed by ion spray mass-spectrometry using a HP 1100 series LC/MSD. The purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography (RP-HPLC) on a $C_{18}$ column, using a 30 minutes linear gradient of 0 to 100% acetonitrile in water and 0.1% trifluoroacetic acid (TFA) at flow rate of 1 ml/min.

*Peptides solutions* – freshly prepared stock solutions were prepared by dissolving the lyophilized form of the peptides in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. To avoid any pre-aggregation, fresh stock solutions were prepared for each experiment. Peptide stock solutions were diluted into microtubes as follows: 5 μL of peptides stock solutions added to 95 μL of 10 mM Tris, pH 7.2 (hence the final concentration of the peptide was 5 mM in the presence of 5% DMSO).

*Congo Red Staining and Birefringence* – A 10 μL suspension of 5mM peptide solution in 10 mM Tris buffer, pH 7.2 aged for 10 days was allowed to dry overnight on a glass microscope slide. Staining was performed by the addition of a 10 μL suspension of saturated Congo Red (CR) and NaCl in 80% ethanol (v/v) solution. The solution was filtered via 0.2 μm filter. The slide was then dried for few hours. Birefringence was determined with a SZX-12 Stereoscope (Olympus, Hamburg, Germany) equipped with cross polarizers. The pictures are magnified ×100.

*Transmission Electron Microscopy* –a 10 μL sample of 5mM peptide solution in 10 mM Tris buffer, pH 7.2 aged for 4 days (for wt peptides) and for 10 days (for

modified peptides) was placed on 400-mesh copper grids (SPI supplies, West Chester PA) covered by carbon-stabilized Formvar film. After 1 minute, excess fluid was removed, and the grid was then negatively stained with 2% uranyl acetate in water for another 2 minutes. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

*Fourier Transform Infrared Spectroscopy* - Infrared spectra were recorded using a Nicolet Nexus 470 FT-IR spectrometer with a DTGS detector. Samples of two weeks aged peptide solutions, were suspended on a $CaF_2$ plate and dried by vacuum. The peptide deposits were resuspended with $D_2O$ and subsequently dried to form thin films. The resuspension procedure was repeated twice to ensure maximal hydrogen to deuterium exchange. The measurements were taken using a 4 $cm^{-1}$ resolution and 2000 scans averaging. The transmittance minima values were determined by the OMNIC analysis program (Nicolet).

*Fourier Transform Infrared Spectroscopy* - IAPP solutions (4 μM peptide in 10 mM Tris buffer pH 7.2), was incubated with and without 40 μM of the various peptides at room temperature. Fibrils formation was assessed by a ten fold diluation of the solutions into a solution that contained 3 μM thioflavin T (ThT) in 50 mM sodium phosphate pH 6.0 and determination of fluorescence at 480 nm with excitation at 450 nm using a Perkin Elmar LS50B spectroflurimeter. As a control a 10 mM Tris buffer pH 7.2 was diluted into the ThT solution and fluorescence was determined as described.

## Results

### Design of peptide based inhibitors

Amyloid fibrils formation is initiated by a site recognition event. We have previously identified a new region within the IAPP molecule that seems to play a major role in this process. The region IAPP(14-20) has a higher affinity to the full-length IAPP than does the widely studied region IAPP(23-29). We chose this domain as a target for inhibitors to bind to, and designed peptides based inhibitors, which are directed to this domain.

We used the design approach by which the native peptide domain serve as a template for small chemical modifications, which maintain the recognition properties of the fragment but abolish the ability to aggregate into amyloid fibrils. By that, when added to a solution of IAPP, the new peptides will bind to the recognition domain, block it and prevent its aggregation.

One way to cancel the aggregation ability of the peptides is to insert elements that are β-sheet breakers, that way the peptides wont be able to display a β-sheet conformation by which the monomers are stacked together to form fibrils. For that purpose we introduce a new methodology of using the α-aminoisobutiric acid residue. α-aminoisobutiric acid (Aib) is an unnatural amino acid that contains two methyl residues attached to $C_\alpha$ of the carboxylic group. Unlike natural amino acids, this molecule does not have a hydrogen atom attached to the $C_\alpha$, this affects widely its sterical properties regarding the $\phi$ and $\psi$ angels of the amide bond. Wile alanine has a wide range of allowed $\phi$ and $\psi$ conformations, Aib, which is a α-methylated alanine has limited $\phi$ and $\psi$ conformations. Its conformational map is derived by the

superposition of the Ramachandran plots of L-alanine and D-alanine (Scheme 1A). As one can see, the allowed angels are limited to small regions and are clearly much more suitable for a α-helix conformation than a β-strand conformation. Hence, this molecule can play the role of preventing β-sheet conformation. Moreover, a comparison of the Ramachandran plots for Aib and proline shows that Aib is a better potent β-sheet breaker than is proline (Scheme 1A).

We designed two peptide-based inhibitors by replacing the alanine and the leucine residues of the ANFLVH and ANFLV fragments, with Aib residues. The new sequences that were form are Aib-NF-Aib-VH and Aib-NF-Aib-V. We first examined the ability of Aib containing peptides to form amyloid fibrils in comparison with the native analogue peptides. Although the chemical changes are very small when comparing the chemical structure of the peptides (Scheme 1B and 1C), we observed critical differences between the native and modified peptides in the ability to aggregate into amyloid fibrils.

**Analysis of the Aib containing peptides**

In order to determine the amiloidogenic nature of the peptides we performed a series of assays including electrone microscopy, birefringence and FT-IR spectroscopy. We examined the aged solutions of the peptides under electronic microscopy (EM) using negative staining. Both native peptides, ANFLVH and ANFLV, formed fibrilar structures with high resembles to the fibrils formed by the full-length IAPP protein. On the other hand, no fibrilar structures were observed for the Aib containing

peptides, Aib-NF-Aib-VH and Aib-NF-Aib-V, even after longer period of incubation, only amorphous aggregates were detected (Figure 1).

Birefringence upon Congo Red staining is very characteristic for amyloids and is another well-used assay. We used the same peptides solutions for congo red staining and examined them under the microscope with cross-polarizers. We observed a tipical yellow-green birefringence for both ANFLVH and ANFLV peptides. Again, in correlation with the EM studies, the peptides Aib-NF-Aib-VH and Aib-NF-Aib-V, exhibited no birefringence and thus seems to have no potential in amyloid formation (Figure 2).

Using FT-IR spectroscopy we were able to get insight about the internal conformation of the observed structures. Upon modification of the native peptides to the Aib containing peptides, a sharp change in the IR spectra was observed. Whereas the ANFLVH and ANFLV peptides spectra were typical β-sheet spectra, with minima at $1630cm^{-1}$ and $1632cm^{-1}$ respectively, the Aib-NF-Aib-VH and Aib-NF-Aib-V peptides displayed minima at $1670cm^{-1}$ and $1666cm^{-1}$, respectively, which are characteristic to a random coil conformation (Figure 3).

Taken together, when we include together all of the experiments, a fundamental difference is observed between the native peptide and the analogues Aib containing peptides. Whereas the native peptides are highly amiloidogenic, the modified Aib containing peptides are not able to form amyloid fibrils. After characterizing these peptides, we next investigated their ability to inhibit the aggregation to amyloid fibrils of the full-length IAPP.

We finally continued to determine the possible inhibition of the various peptide studied on full-length IAPP molecule. To that end IAPP was incubated with the studied peptide and the amount of amyloid in the solution were determined using ThT fluorescence. The result clearly show an inhibition effect of all peptides as compared to the wild-type peptide alone (Figure 4)

**Discussion**

Here we demonstrated that a very minor change in short peptides (Scheme 1) completely abolish their ability to form amyloid fibrils. An addition of a methyl group in a single or multiple places along the polypeptide chain blocked the strcutral transition that is associated with amyloid fibrils formation. Both our qualitative analysis using electron microscopy (Figure 1) and Congo Red birefringence (Figure 2) as well as structural analysis using FT-IR (Figure 3) clearly revealed the complete transition that caused by this change. Furthermore, our mechanistic understanding of the process of amyloid fibrils formation allowed us the design inhibtor peptides that are based on this principle. The modification of the IAPP molecular recogntion and self-assembly region (Mazor et al, 2002) with Aib moieties resulted in a very significant inhibition of amyloid fibrils formation by the full length IAPP.

Our methods is not limited only to IAPP or only to Aib. We claim that such a modification of any molecular recognition and/or self-assembly domain in each of the known amyloid forming polypeptide with Aib or any other Cα methylated amino-acid will result in the production of efficient inhibitory molecules

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## Figure legends

**Scheme 1: Ramachandran plot and the studied peptides.** Schematic representation of the Aib residue and its limitation on amyloid formation. (a) Ramachandran plot showing the sterically allowed regions for all residues (yellow for fully allowed, orange for partially allowed), for L-Proline (blue) and for the achiral Aib residue (magenta). (b) The chemical structure of the longer wt peptide (ANFLVH), functional groups, which are to be modified are marked in blue. (c) The chemical structure of the longer modified peptide (Aib-NF-Aib-VH), modified groups are marked in red.

**Figure 1: Electron microscopy**. Electron microscopic examination of insoluble aggregates of wt and modified peptides formed in an aged solution: **(a)** ANFLVH, **(b)** ANFLV, **(c)** Aib-NF-Aib-VH, **(d)** Aib-NF-Aib-V.The scale bar represents 100 nm. . Peptide solutions were used as described in the "Materials and Methods" section.

**Figure 2: Congo red binding assay**. Examination of the amyloidogenic nature of wt and modified peptides by congo red binding. Microscopic examination under polarized light following staining with CR of the peptides: **(A)** ANFLVH, **(B)** ANFLV, **(C)** Aib-NF-Aib-VH, **(D)** Aib-NF-Aib-V . Peptide solutions were aged for 11 days as described in the "Materials and Methods" section.

**Figure 3: Fourier transform infrared spectroscopy.** Analysis of secondary structures of insoluble aggregates of hIAPP fragments formed in an aged solution as assessed by fourier transform infrared spectroscopy (FT-IR): (a) wt peptide ANFLVH and modified peptide Aib-NF-Aib-V, as pointes by arrows. (b) wt peptide ANFLVH

and modified peptide Aib-NF-Aib-V, as pointed by arrows. Peptide solutions described in the "Materials and Methods" section.

**Figure 4: Inhibition assay.** Analysis of inhibitory effect of the peptide on amyloid fibrils formation by IAPP. IAPP was incubated alone or with the various peptides studied. The amount of fibrils in each experiment as various time points was determined using ThT fluorescence.

Claims:

1. A method of inhibing amyloid fibrils formation by at least one alpha-aminoisobutyric acid substituted analogue, essentially as described and exemplified herein.

2. An alpha-aminoisobutyric acid substituted analogue, essentially as described and exemplified herein.

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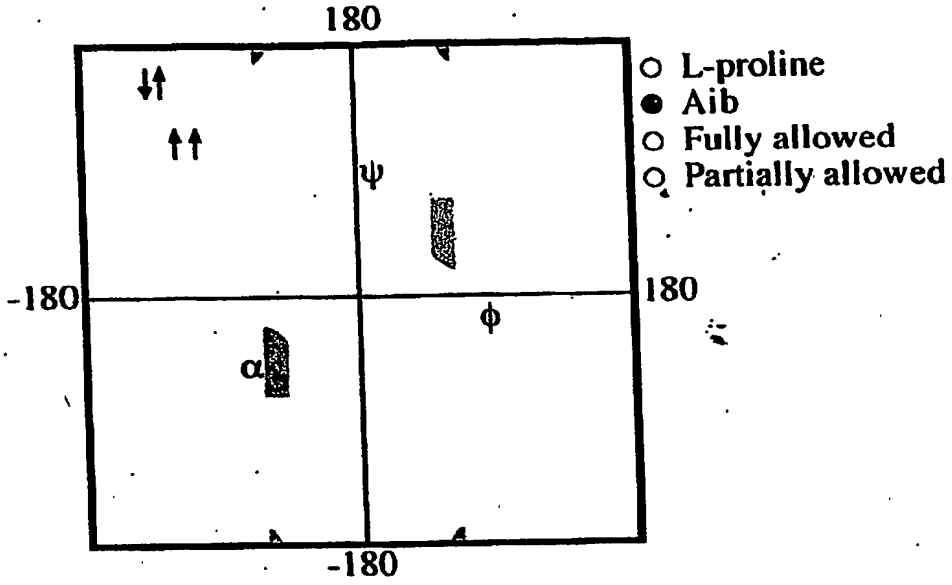
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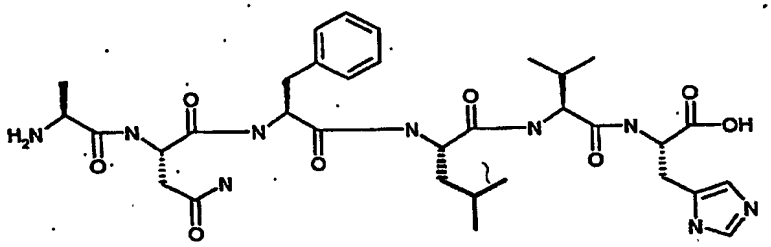

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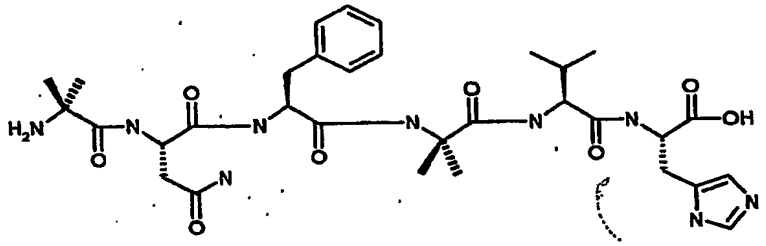

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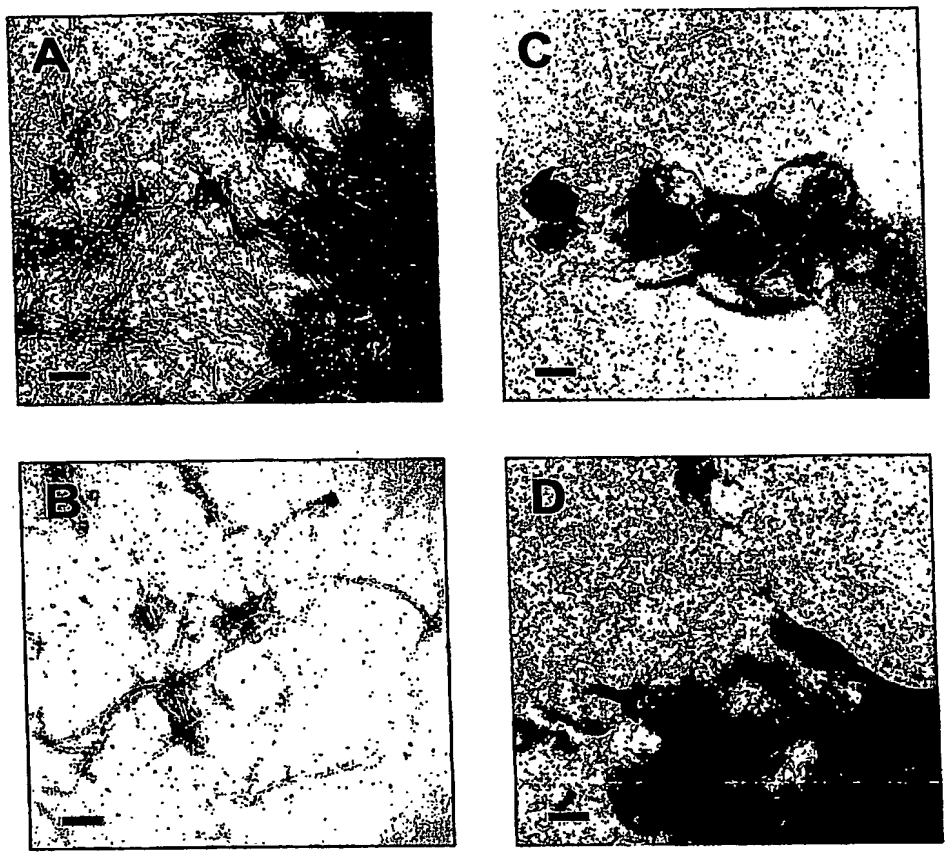


**Figure 1**

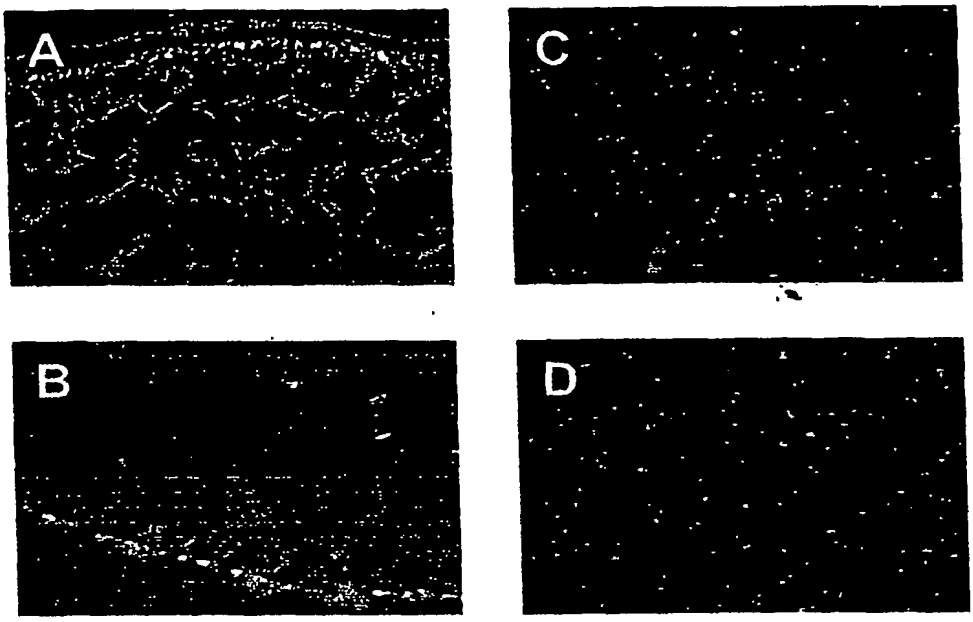


**Figure 2**

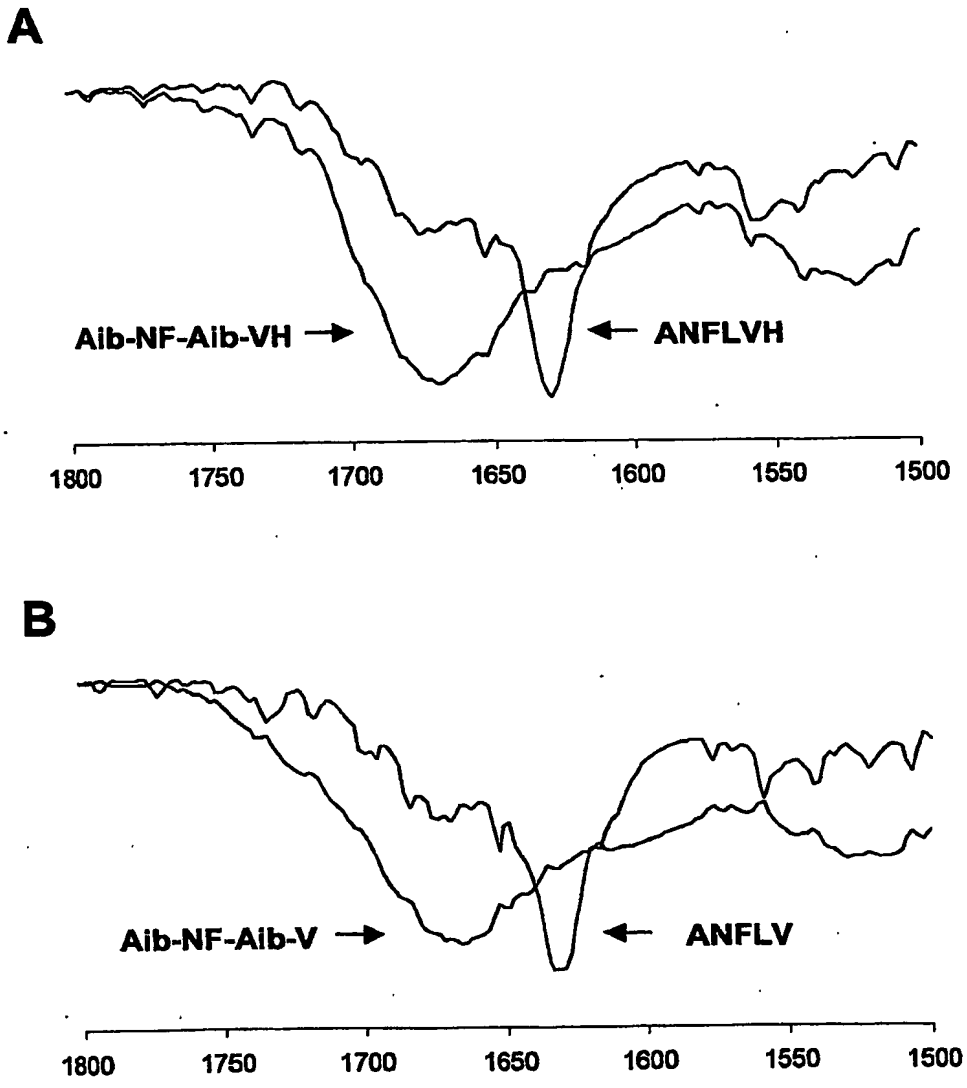
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**Figure 3**

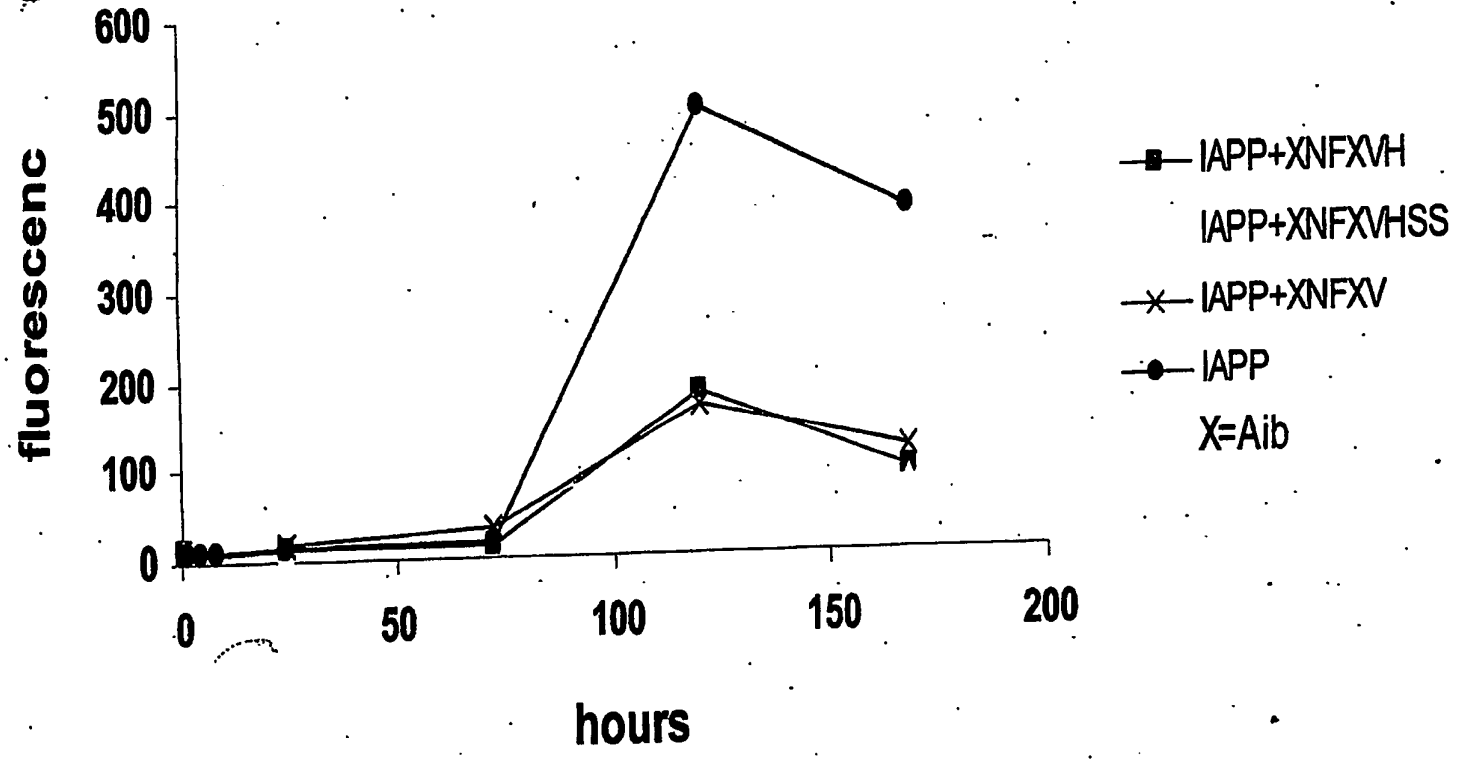
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Figure 4